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X-ray Holographic Microscopy of Biological Specimens with an Electronic Zooming Tube

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X-ray holographic microscopy of biological specimens has been performed using in-line holography with the following improvements: (i) an electronic zooming tube is utilized as a detector, and (ii) the object planes are restricted by a metal frame which is not transparent to X-rays at the wavelengths used in the present experiments. The latter modification is useful in computer reconstruction and serves as a reference area where there are no photons in the reconstructed images. This system can minimize the inclusion of errors when recording and reading out holograms compared with methods using an X-ray resist as a detector, or optical reconstruction methods. Two-dimensional images of human cells have been successfully obtained with the present system. Comparison of these images with optical images revealed a good coincidence in structure outlines but not in internal structure images. Holographic observation of HeLa cells at different wavelengths showed a wavelength dependence of the reconstructed images. Since the electronic zooming tube is applicable to a wide range of X-ray wavelengths and has a promising potential to improve its resolution, these results strongly suggest that the present system is a good candidate for the development and establishment of an X-ray holographic microscopy system.

Keywords: HeLa cells; undulator radiation; wavelength dependency; holography; imaging; X-ray zooming tube.

1. Introduction

X-ray microscopy of biological specimens has been extensively developed in recent years (see *e.g.* Shinohara, Yada, Kihara & Saito, 1990; Michette, Morrison & Buckley, 1992; Jacobsen & Trebes, 1993). For instance, X-ray contact microscopy (Shinohara, Ito & Kinjo, 1994; Kinjo *et al.*, 1994), imaging microscopy (Schmahl *et al.*, 1991; Guttmann *et al.*, 1992) and scanning microscopy (Kirz, 1991; Williams, Jacobsen, Kirz, Lamm & Van't Hof, 1992) have already been developed and applied to observe hydrated biological specimens. However, none of these microscopies will be applicable to three-dimensional observations of thick hydrated biological specimens for the following reasons: (i) thick specimens cannot be observed at high resolution by contact microscopy because of Fresnel diffraction; (ii) image blurring by thermal diffusion (Ito & Shinohara, 1992) and the induction of radiation damage (Shinohara & Ito, 1991) require short exposure times and make multiple exposures impossible for high-resolution imaging unless the specimens are fixed; and (iii) more than two exposures are essential for stereo imaging with imaging microscopes or scanning microscopes. In these respects, X-ray holography is a unique candidate for three-dimensional imaging of thick hydrated biological specimens at high resolution since holography with a single-shot exposure of short-pulsed coherent X-rays, such as an X-ray laser, may fulfill the condition for three-dimensional imaging.

X-ray holographic microscopy of biological specimens has been described by several groups. Aoki & Kikuta (1974) showed X-ray holograms and reconstructed images of erythrocytes for the first time. Solem (1986) has estimated the energy absorbed by the specimen. London, Rosen & Trebes (1989) studied the absorbed energy and proposed effective wavelengths for the holography of biological molecules. Joyeux, Lowenthal, Polack & Bernstein (1988) recorded holograms of diatoms with a photoresist and reconstructed them optically. Howells, Jacobsen and their colleagues successfully obtained holograms at a resolution of 40 nm with X-ray resists and obtained computer-

reconstructed images at a resolution of 80 nm (Howells *et al.*, 1987; Jacobsen *et al.*, 1988; Jacobsen, Howells, Kirz & Rothman, 1990). However, these methods can all have some limitations in producing highly faithful reconstructions because the following steps can cause the inclusion of errors: (i) optical reconstruction requires the methods to overcome difficulties such as optical noise including twin-image problems; and (ii) reconstruction of holograms on a resist by a computer requires many steps to read out the holograms and to digitize the data. This problem of error inclusion may not affect the system reported by McNulty *et al.* (1992), but their system is limited in resolution and in applicable wavelengths, and needs many more photons due to the use of a zone plate, which may be critical for high-resolution imaging.

In the present communication, we have utilized an electronic zooming tube (Kinoshita *et al.*, 1992, 1993; Watanabe *et al.*, 1993) for recording holograms as digitized data and this enabled us to reconstruct images directly with the computer, and to succeed in imaging dried HeLa cells two-dimensionally. Wavelength-dependent images of HeLa cells were also obtained. These results suggest that this system is most effective for holographic microscopy.

2. Materials and methods

Fig. 1 shows the experimental arrangement for recording holograms. Undulator radiation from the BL-2B experimental station at the Photon Factory, National Laboratory for High Energy Physics, Japan, in single-bunch-mode operation was used through a 10 m grazing-incidence monochromator (Yagishita, Masui, Toyoshima, Maezawa & Shigemasa, 1992). The wavelength (λ) chosen was 4.48 or 3.26 nm and the full width at half maximum (FWHM) of the X-rays ($\Delta\lambda$) was set to 0.004 nm by an exit slit. A pinhole with a diameter of 50 μm was placed at the focal point of a refocusing mirror located downstream of the exit slit. The pinhole, specimen and detector were aligned as shown in Fig. 1, and the distance between pinhole and detector was 2135 mm. The specimens were placed in front of the detector at a distance of 10.1 mm. The distance was measured with an error of 0.01–0.02 mm. For the detector, we utilized an electronic zooming tube (Hamamatsu Photonics KK, Japan) (Kinoshita *et al.*, 1992, 1993). In the electronic zooming tube, photoelectrons converted by the CsI photocathode are enlarged electromagnetically and collected with multichannel plates (MCP). Photons per pixel from the multichannel plates were detected two-dimensionally with a CCD camera and stored by an image processor (ARGUS-50, Hamamatsu Photonics KK, Japan) in an array of 512 × 483 pixels. The recording time was 5–80 min. The stored digital data are linearly correlated with the number of photons at the surface of the photocathode.

The following biological specimens were observed: HeLa cells (derived from human cervical cancer) grown on a formvar membrane supported by a gold EM-grid (200 mesh), fixed with 2.5% glutaraldehyde and critical point dried.

The photon intensity was monitored by photoelectrons emitted from a gold photocathode (thickness 100 nm) inserted 290 mm downstream from the pinhole. The intensities of an illuminated area of size *ca* 0.25 × 2.5 mm on the

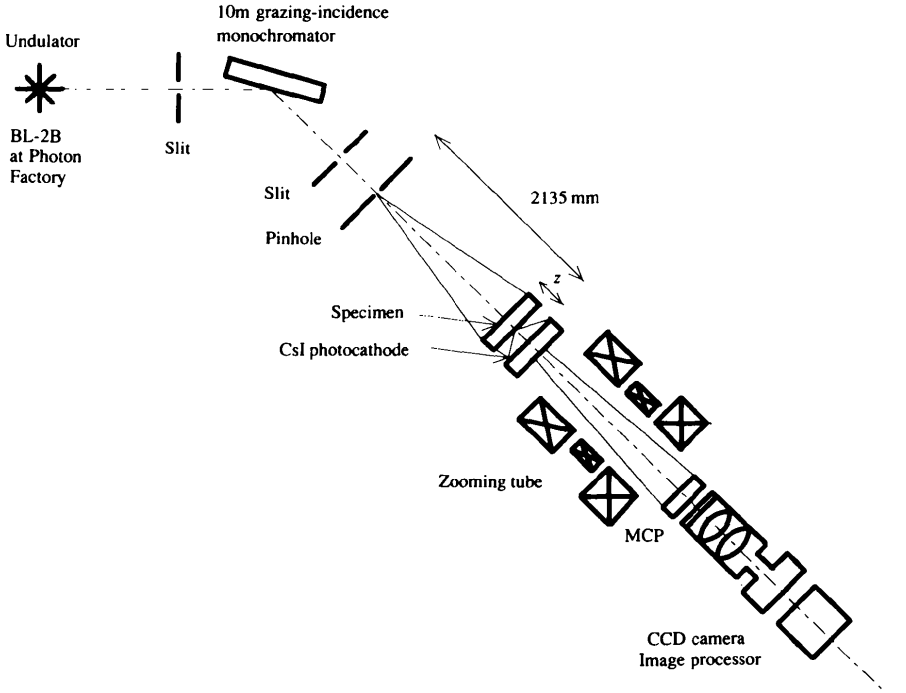

Figure 1
Schematic view of the recording system for in-line X-ray holography.

CsI photocathode in the present experiments were estimated using the electron photoemission yield reported by Henke, Knauer & Premaratne (1981).

Recorded holograms were reconstructed with a computer by the iteration method using the Fresnel transform–inverse Fresnel transform. Basically, our image reconstruction algorithm is the same as that of Jacobsen *et al.* (1990) with additional assumptions for the object plane. These additional assumptions are as follows: (i) the object does not affect the phase of the illuminated X-rays; (ii) the aperture of the object plane has a known shape, dimension and position; and (iii) intensity distributions for illuminating the object plane can be estimated roughly by those of the observed Fresnel pattern. It should be noted that no assumptions were made for absorption by the object.

The second assumption was defined by the experimental conditions with the frame of the EM-grid. Moreover, we provided the initial phase distributions at the observation plane. The distributions can be calculated from the Fresnel diffraction of the known aperture of the object plane. The data flow of the iteration algorithm is shown in Fig. 2. A more precise algorithm has been presented elsewhere (Kodama, 1994; Kodama *et al.*, 1995). The iteration was performed 30 times.

3. Results

Fig. 3 shows a hologram of a HeLa cell recorded at 4.48 nm (Fig. 3*a*) and its reconstructed image (Fig. 3*b*). The reconstruction successfully revealed the sharp outline of the cell with the inner structure clearly resolved. Fig. 4 shows an enlarged picture of the reconstructed image shown in Fig. 3(*b*) and an image observed by optical microscopy. The reconstructed image (Fig. 4*a*) clearly shows a nucleus with cytoplasm comparable with the phase-contrast optical image (Fig. 4*b*). More than three fibre-like structures (0.6–0.7 μm in diameter) are seen inside the cytoplasm on the right end of the cell in the reconstructed image. Some of the inner structures in the reconstructed image do not correspond to the structures seen in the optical image. This apparent difference in the images may be caused by the difference in the contrast mechanisms of the two types of microscopy. In addition, the X-ray image shows nuclear internal structures. The significance of these structures seen in the X-ray image remains to be clarified.

Fig. 5 shows the wavelength dependence of the holograms of HeLa cells recorded at 4.48 nm (Fig. 5*a*) and 3.26 nm (Fig. 5*b*), and their corresponding reconstructed images (Figs. 5*c*,*d*). The fringe pattern appears to be similar for the holograms at 4.48 and 3.26 nm. Both of the reconstructed images show some internal structures, but there are many differences between them. Since the wavelength at 4.48 nm is slightly longer than that of the absorption edge of carbon, the absorption of carbon must contribute less to the contrast at 4.48 nm (Fig. 5*c*). Similarly, the absorption of nitrogen must contribute less to the contrast at 3.26 nm (Fig. 5*d*).

4. Discussion

We have succeeded in X-ray holography of biological specimens by recording holograms with an electronic zooming tube and by reconstructing them with a computer program. In this system, holograms are recorded as digitized data, which allows direct reconstruction with a computer. Real-time imaging by X-ray holography may be possible in the future using this method.

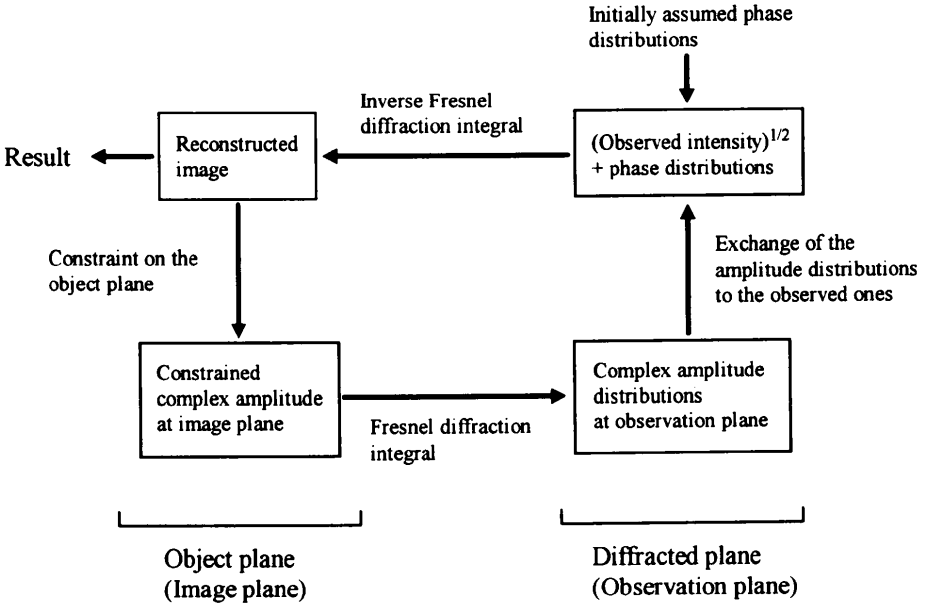

Figure 2
The data flow for iteration for image reconstruction.

The resolution of the images (*ca* 1 μm) observed by X-ray holography was not as good as those obtained with optical microscopy. Nevertheless, the present system offers strong possibilities for improved resolution. The main factors responsible for the improvement of resolution are (i) the optical system and coherence requirement, and (ii) the resolution power of the detector.

The resolution is limited by the coherence of X-rays described by the following equations:

$$\mu^2 \geq 2\lambda z/r = 2\Delta\lambda z \quad (1)$$

$$\mu \geq 4\theta z \quad (2)$$

where μ refers to the expected resolution, z is the distance between the specimen and the detector, $r = \lambda/\Delta\lambda$, and θ refers to the angle divergence of the source from the specimen. In the present experiments, z = 10.1 mm, $\Delta\lambda$ = 0.004 nm and θ = 50 μm (pinhole diameter)/2135 mm (pinhole–specimen distance). Therefore, the resolution $\mu \geq$ 0.95 μm.

We have used an electronic zooming tube as a two-dimensional detector. The idea is in accord with the comments by Mueller & Jorna (1977). With this detector, the two-dimensional distribution of photon intensities are recorded as digitized data. The data can be directly reconstructed with a computer and, hence, the inclusion of artefacts during reconstruction must be minimized. The resolution of the electronic zooming tube is reported as 0.2–0.3 μm (Kinoshita *et al.*, 1992) and

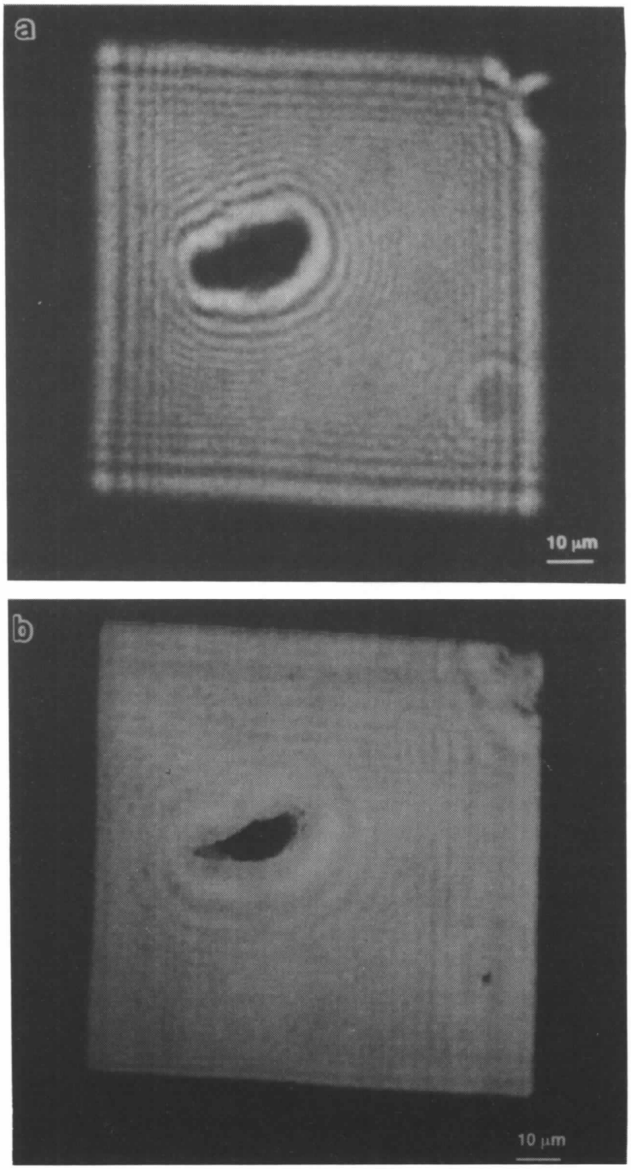

Figure 3
X-ray hologram of a HeLa cell at a wavelength of 4.48 nm and its reconstructed image. (*a*) Digitized hologram recorded by an electronic zooming tube. The exposure time was 60 min (2.93×10^{12} photons cm^{-2}). (*b*) Reconstructed image after 30 iterations in the reconstruction algorithm.

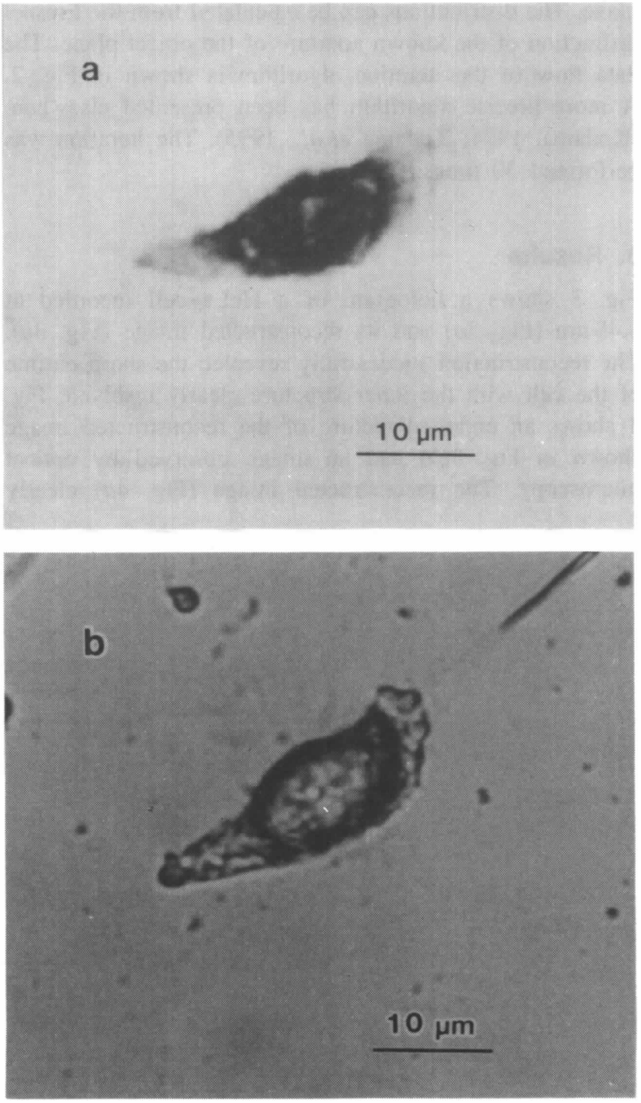

Figure 4
Reconstructed image of a HeLa cell recorded at 4.48 nm and the optical image of the same specimen. (*a*) Enlarged picture of the reconstructed image shown in Fig. 3(*b*). (*b*) Optical image observed by phase-contrast microscopy.

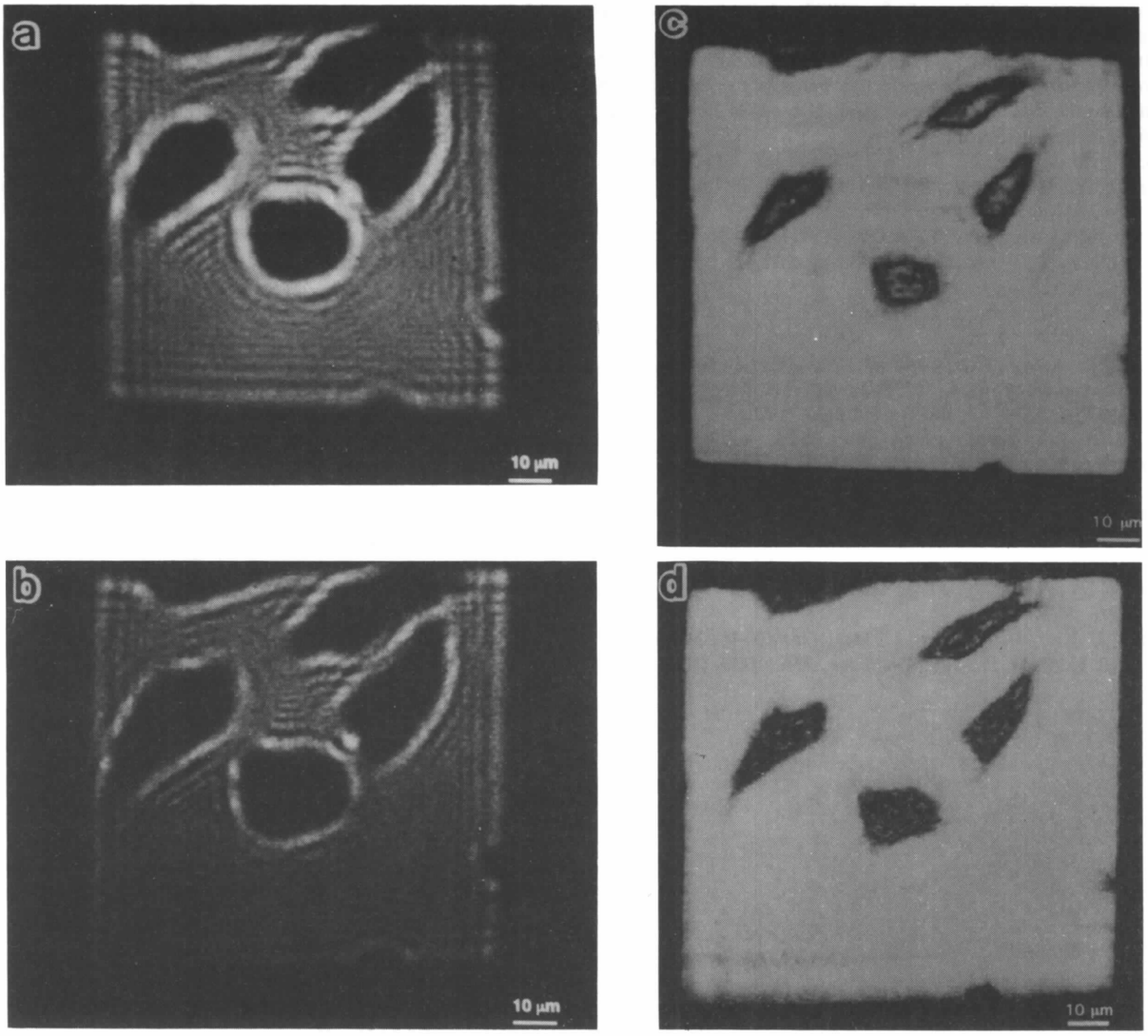

Figure 5
Wavelength dependence of X-ray holograms of HeLa cells [(*a*) and (*b*)] and their reconstructed images [(*c*) and (*d*)]. The first harmonic of the undulator radiation was used for 4.48 nm [(*a*) and (*c*)] and 3.26 nm [(*b*) and (*d*)]. Reconstructed images were obtained after 30 iterations. The exposure times were (*a*) 80 min (6.11×10^{12} photons cm^{-2}), and (*b*) 5 min (2.39×10^{12} photons cm^{-2}).

remains to be improved in the future. The upper limit of resolution may theoretically be on the atomic scale of the photocathode if a proper substrate is chosen, although the efficiency would probably be very low in such a case.

For three-dimensional observation by holography with a single-shot exposure, a detector with very high resolution will be required. Tomographic X-ray holography (McNulty *et al.*, 1993) may be an alternative method. In the observation of hydrated specimens by tomographic holography, simultaneous exposures by multiple pulsed X-ray sources of different angles would be required. The present system may also be directly applicable for this purpose.

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